

IN THE SPECIFICATION

I. Abstract

Please replace the original Abstract with the following paragraph:

Disclosed herein are methods for treating lectin-mediated platelet disorders. The methods comprise administering a pharmaceutically effective amount of a nucleic acid ligand to a lectin. Also disclosed are methods for treating lectin-mediated inflammation or lymphocyte tracking disorders comprising administering a pharmaceutically effective amount of a nucleic acid ligand to lectin. Exemplary lectins contemplated herein include wheat germ agglutinin, L-selectin, E-selectin, and P-selectin.

II. Specification

Please replace paragraph [0005] with the following amended paragraph:

[0005] The diversity of lectin mediated functions provides a vast array of potential therapeutic targets for lectin antagonists. Both lectins that bind endogenous carbohydrates and those that bind exogenous carbohydrates are target candidates. For example, antagonists to the mammalian ~~selectin~~ selecting, a family of endogenous carbohydrate binding lectins, may have therapeutic applications in a variety of leukocyte-mediated disease states. Inhibition of selectin binding to its receptor blocks cellular adhesion and consequently may be useful in treating inflammation, coagulation, transplant rejection, tumor metastasis, rheumatoid arthritis, reperfusion injury, stroke, myocardial infarction, burns, psoriasis, multiple sclerosis, bacterial sepsis, hypovolaemic and traumatic shock, acute lung injury, and ARDS.

Please replace paragraph [0006] with the following amended paragraph:

[0006] The ~~selectin~~ selecting, E-, P- and L-, are three homologous C-type lectins that recognize the tetrasaccharide, sialyl-Lewis^X (C. Foxall et al, 1992, J. Cell Biol. 117,895-902). Selectins mediate the initial adhesion of neutrophils and monocytes to activated vascular endothelium at sites of inflammation (R. S. Cotran et al., 1986, J. Exp. Med. 164, 661-; M. A. Jutila et al., 1989, J. Immunol. 143, 3318-; J. G. Geng et al., 1990, Nature, 757; U. H. Von Adrian et al., 1994, Am. J. Physiol. Heart Circ. Physiol. 263, H1034-H1044). In addition, L-selectin is responsible for the homing of lymphocytes to peripheral and mesenteric lymph nodes (W. M. Gallatin et al., 1983, Nature 304, 30; T. K. Kishimoto et al., 1990, Proc. Natl. Acad. Sci. 87, 2244-) and P-selectin

mediates the adherence of platelets to neutrophils and monocytes (S- C. Hsu-Lin et al., 1984, J. Biol. Chem. 259, 9121).

Please replace paragraph [0009] with the following amended paragraph:

[0009] Antagonists to lectins that recognize exogenous carbohydrates may have wide application for the prevention of infectious diseases. Many viruses (influenza A, B and C; Sendhi, Newcastle disease, coronavirus, rotavirus, encephalomyelitis virus, encephalomyocarditis virus, reovirus, paramyxovirus) use lectins on the surface of the viral particle for attachment to cells, a prerequisite for infection; antagonists to these lectins are expected to prevent infection (A. Varki, 1993, Glycobiol.3, 97-130). Similarly colonization/infection strategies of many bacteria utilize cell surface lectins to adhere to mammalian cell surface glyco-conjugates. Antagonists to bacterial cell surface lectins are expected to have therapeutic potential for a wide spectrum of bacterial infections, including: gastric (*Helicobacter pylori*), urinary tract (*E. coli*), pulmonary (*Klebsiella pneumoniae*, ~~*Streptococcus*~~ *Streptococcus pneumoniae*, *Mycoplasma pneumoniae*) and oral (*Actinomyces naeslundii* and *Actinomyces viscosus*) colonization/infection (S. N. Abraham, 1994, Bacterial Adhesins, in The Handbook of Immunopharmacology: Adhesion Molecules, C. D. Wegner, ed; B. J. Mann et al., 1991, Proc. Natl. Acad. Sci. 88, 3248-3252). A specific bacterial mediated disease state is *Pseudomonas aeruginosa* infection, the leading cause of morbidity and mortality in cystic fibrosis patients. The expectation that high affinity antagonists will have efficacy in treating *P. aeruginosa* infection is based on three observations. First, a bacterial cell surface, GalNAc β 1-4Gal binding lectin mediates infection by adherence to asialogangliosides α GM1 and α GM2) of pulmonary epithelium (L. Imundo et al., 1995, Proc. Natl. Acad. Sci 92, 3019-3023). Second, in vitro, the binding of *P. aeruginosa* is competed by the gangliosides' tetrasaccharide moiety, Gal β 1-3GalNAc β 1-4Gal β 1-4Glc. Third, in vivo, instillation of antibodies to *Pseudomonas* surface antigens can prevent lung and pleural damage (J. F. Pittet et al., 1993, J. Clin. Invest. 92, 1221-1228).

Please replace paragraph [0010] with the following amended paragraph:

[0010] Non-bacterial microbes that utilize lectins to initiate infection include *Entamoeba histalytica* (a Gal specific lectin that mediates adhesion to intestinal mucosa; W. A. Petri, Jr., 1991, AMS News 57:299-306) and *Plasmodium faciparum* (a lectin specific for the terminal

Neu5Ac(a2-3)Gal of glyophorin A of ~~erythrocytes~~ ~~orthrocytes~~; P. A. Orlandi et al., 1992, J. Cell Biol. 116:901-909). Antagonists to these lectins are potential therapeutics for dysentery and malaria.

Please replace paragraph [0012] with the following amended paragraph:

[0012] There are still other conditions for which the role of lectins is currently speculative. For example, genetic mutations result in reduced levels of the serum mannose-binding protein (MBP). Infants who have insufficient levels of this lectin suffer from severe infections, but adults do not. The high frequency of mutations in both Oriental ~~oriental~~ and Caucasian populations suggests a condition may exist in which low levels of serum mannose-binding protein are advantageous. Rheumatoid arthritis (RA) may be such a condition. The severity of RA is correlated with an increase in IgG antibodies lacking terminal galactose residues on Fc region carbohydrates (A. Young et al., 1991, Arth. Rheum. 34, 1425-1429; I. M. Roitt et al., 1988, J. Autoimm. 1, 499-506). Unlike their normal counterpart, these gal-deficient carbohydrates are substrates for MBP. MBP/IgG immunocomplexes may contribute to host tissue damage through complement activation. Similarly, the eosinophil basic protein is cytotoxic. If the cytotoxicity is mediated by the lectin activity of this protein, then a lectin antagonist may have therapeutic applications in treating eosinophil mediated lung damage.

Please replace paragraph [0015] with the following amended paragraph:

[0015] Although lectin specificity is usually expressed in terms of monosaccharides and/or oligosacchrides (i.e., MBP binds mannose, fucose and N-acetylglucosamine), the affinity for monosaccharides is weak. The dissociation constants for monomeric saccharides are typically in the millimolar range (Y. C. Lee, 1992, FASEB J. 6:3193-3200; G. D. Glick et al., 1991, J Biol.Chem. 266:23660-23669; Y. Nagata and M. M. Burger, 1974, J. Biol. Chem. 249:3116 [[116]]-3122).

Please replace paragraph [0017] with the following amended paragraph:

[0017] The affinity of the mannose-binding protein and other lectins for their natural ligands is greater than that for monosaccharides. Increased specificity and affinity can be accomplished by establishing additional contacts between a protein and its ligand (K. Drickamer, 1993, supra) either by 1) additional contacts with the terminal sugar (i.e., chicken hepatic lectin binds N-

acetylglucosamine N-acetylglucose amine with greater affinity than mannose or fucose suggesting interaction with the 2-substituent); 2) clustering of CRDs for binding complex oligosaccharides (i.e., the mammalian asialoglycoprotein receptor); 3) interactions with additional saccharide residues (i.e., the lectin domain of selectins appears to interact with two residues of the tetrasaccharide sialyl-Lewis^X: with the charged terminal residue, sialic acid, and with the fucose residue; wheat germ agglutinin appears to interact with all three residues of trimers of N-acetylglucosamine); or by 4) contacts with a non-carbohydrate portion of a glycoprotein.

Please replace paragraph [0024] with the following amended paragraph:

[0024] A method for the in vitro evolution of nucleic acid molecules with highly specific binding to target molecules has been developed. This method, Systematic Evolution of Ligands by EXponential enrichment, termed SELEX, is described in U.S. patent application Ser. No. 07/536,428, entitled "[37] Systematic Evolution of Ligands by Exponential Enrichment," now abandoned, U.S. patent application Ser. No. 07/714,131, filed Jun. 10, 1991, entitled "Nucleic Acid Ligands," now U.S. Pat. No. 5,475,096, U.S. patent application Ser. No. 07/931,473, filed Aug. 17, 1992, entitled "Methods for Identifying Nucleic Acid Ligands," now U.S. Pat. No. 5,270,163 (see also PCT/US91/04078), each of which is herein specifically incorporated by reference. Each of these applications, collectively referred to herein as the SELEX Patent Applications, describes a fundamentally novel method for making a nucleic acid ligand to any desired target molecule.

Please replace paragraph [0026] with the following amended paragraph:

[0026] The basic SELEX method has been modified to achieve a number of specific objectives. For example, U.S. patent application Ser. No. 07/960,093, filed Oct. 14, 1992, entitled "Method for Selecting Nucleic Acids on the Basis of Structure," describes the use of SELEX in conjunction with gel electrophoresis to select nucleic acid molecules with specific structural characteristics, such as bent DNA. U.S. patent application Ser. No. 08/123,935, filed Sep. 17, 1993, entitled "Photoselection of Nucleic Acid Ligands" describes a SELEX based method for selecting nucleic acid ligands containing photoreactive groups capable of binding and/or photocrosslinking to and/or photoinactivating a target molecule. U.S. patent application Ser. No.

08/134,028, filed Oct. 7, 1993, entitled "High-Affinity Nucleic Acid Ligands That Discriminate Between Theophylline and Caffeine," describes a method for identifying highly specific nucleic acid ligands able to discriminate between closely related molecules, termed Counter-SELEX. U.S. patent application Ser. No. 08/143,564, filed Oct. 25, 1993, entitled "Systematic Evolution of Ligands by EXponential Enrichment: Solution SELEX," describes a SELEX-based method which achieves highly efficient partitioning between oligonucleotides having high and low affinity for a target molecule. U.S. patent application Ser. No. 07/964,624, filed Oct. 21, 1992, entitled "~~Methods of Producing~~ Nucleic Acid Ligands to HIV-RT and HIV-1 REV," now U.S. Patent No. 5,496,938, describes methods for obtaining improved nucleic acid ligands after SELEX has been performed. U.S. patent application Ser. No. 08/400,440, filed Mar. 8, 1995, entitled "Systematic Evolution of Ligands by EXponential Enrichment: Chemi-SELEX," now U.S. Patent No. 5,705,337, describes methods for covalently linking a ligand to its target.

Please replace paragraph [0027] with the following amended paragraph:

[0027] The SELEX method encompasses the identification of high-affinity nucleic acid ligands containing modified nucleotides conferring improved characteristics on the ligand, such as improved in vivo stability or improved delivery characteristics. Examples of such modifications include chemical substitutions at the ribose and/or phosphate and/or base positions. SELEX-identified nucleic acid ligands containing modified nucleotides are described in U.S. patent application Ser. No. 08/117,991, filed Sep. 8, 1993, entitled "High Affinity Nucleic Acid Ligands Containing Modified Nucleotides," that describes oligonucleotides containing nucleotide derivatives chemically modified at the 5- and 2'-positions of pyrimidines. U.S. patent application Ser. No. 08/134,028, *supra*, describes highly specific nucleic acid ligands containing one or more nucleotides modified with 2'-amino (2'-NH₂), 2'-fluoro (2'-F), and/or 2'-O-methyl (2'-OMe). U.S. patent application Ser. No. 08/264,029, filed Jun. 22, 1994 (now abandoned), entitled "Novel Method of Preparation of Known and Novel 2'-Modified Nucleosides by 2' Modified Pyrimidine Intramolecular Nucleophilic Displacement," describes novel methods for making 2'-modified nucleosides.

Please replace paragraph [0028] with the following amended paragraph:

[0028] The SELEX method encompasses combining selected oligonucleotides with other selected oligonucleotides as described in U.S. patent application Ser. No. 08/284,063, filed Aug. 2, 1994, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Chimeric SELEX," now U.S. Patent No. 5,637,459. The SELEX method also includes combining the selected nucleic acid ligands with non-oligonucleotide functional units and U.S. patent application Ser. No. 08/234,997, filed Apr. 28, 1994, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Blended SELEX," now U.S. Patent No. 5,683,867, and U.S. patent application Ser. No. 08/434,465, filed May 4, 1995, entitled "Nucleic Acid Ligand Complexes," now U.S. Patent No. 6,011,020. These applications allow the combination of the broad array of shapes and other properties, and the efficient amplification and replication properties, of oligonucleotides with the desirable properties of other molecules. Each of the above described patent applications which describe modifications of the basic SELEX procedure are specifically incorporated by reference herein in their entirety.

Please replace paragraph [0036] with the following amended paragraph:

[0036] FIG. 1 shows consensus hairpin secondary structures for WGA 2'-NH₂ RNA ligands: (a) family 1 (SEQ ID NO: 391), (b) family 2 (SEQ ID NO: 392) and (c) family 3 (SEQ ID NO: 393). Nucleotide sequence is in standard one letter code. Invariant nucleotides are in bold type. Nucleotides derived from fixed sequence are in lower case.

Please replace paragraph [0047] with the following amended paragraph:

[0047] FIG. 12 shows the consensus hairpin secondary structures for family 1 ssDNA ligands to L-selectin (SEQ ID NO: 394). Nucleotide sequence is in standard one letter code. Invariant nucleotides are in bold type. The base pairs at highly variable positions are designated N--N'. To the right of the stem is a matrix showing the number of occurrences ~~occurrences~~ of particular base pairs for the position in the stem that is on the same line.

Please replace paragraph [0050] with the following amended paragraph:

[0050] FIG. 15 shows the consensus hairpin secondary structures for 2'-F RNA ligands to L-selectin (SEQ ID NO: 395). Nucleotide sequence is in standard one letter code. Invariant nucleotides are in bold type. The base pairs at highly variable positions are designated N-N'. To

the right of the stem is a matrix showing the number of occurrences ~~occurences~~ of particular base pairs for the position in the stem that is on the same line.

Please replace paragraph [0051] with the following amended paragraph:

[0051] FIG. 16 shows the consensus hairpin secondary structures for 2'-F RNA ligands to P-selectin (SEQ ID NO: 396). Nucleotide sequence is in standard one letter code. Invariant nucleotides are in bold type. The base pairs at highly variable positions are designated N-N'. To the right of the stem is a matrix showing the number of occurrences ~~occurences~~ of particular base pairs for the position in the stem that is on the same line.

Please replace paragraph [0052] with the following amended paragraph:

[0052] This application describes high-affinity nucleic acid ligands to lectins identified through the method known as SELEX. SELEX is described in U.S. patent application Ser. No. 07/536,428, entitled "Systematic Evolution of Ligands by EXponential Enrichment", now abandoned; U.S. patent application Ser. No. 07/714,131, filed Jun. 10, 1991, entitled "Nucleic Acid Ligands", now U.S. Pat. No. 5,475,096; and U.S. patent application Ser. No. 07/931,473, filed Aug. 17, 1992, entitled "Methods for Identifying Nucleic Acid Ligands", now U.S. Pat. No. 5,270,163, (see also PCT/US91104078). These applications, each specifically incorporated herein by reference, are collectively called the SELEX Patent Applications.

Please replace paragraph [0060] with the following amended paragraph:

[0060] This invention also includes the ligands as described above, wherein certain chemical modifications are made in order to increase the in vivo stability of the ligand or to enhance or mediate the delivery of the ligand. Examples of such modifications include chemical substitutions at the sugar and/or phosphate and/or base positions of a given nucleic acid sequence. See, e.g., U.S. patent application Ser. No. 08/117,991, filed Sep. 8 [[9]], 1993, entitled "High Affinity Nucleic Acid Ligands Containing Modified Nucleotides" which is specifically incorporated herein by reference. Additionally, in co-pending and commonly assigned U.S. patent application Ser. No. 07/964,624, filed Oct. 21, 1992 ('624), now U.S. Pat. No. 5,496,938, methods are described for obtaining improved nucleic acid ligands after SELEX has been performed. The '624 application, entitled "~~Methods of Producing~~ Nucleic Acid Ligands to HIV-

RT and HIV-1 rev," is specifically incorporated herein by reference. Further included in the '624 patent are methods for determining the three-dimensional structures of nucleic acid ligands. Such methods include mathematical modeling and structure modifications of the SELEX-derived ligands, such as chemical modification and nucleotide substitution. Other modifications are known to one of ordinary skill in the art. Such modifications may be made post-SELEX (modification of previously identified unmodified ligands) or by incorporation into the SELEX process. Additionally, the nucleic acid ligands of the invention can be complexed with various other compounds, including but not limited to, lipophilic compounds or non-immunogenic, high molecular weight compounds. Lipophilic compounds include, but are not limited to, cholesterol, dialkyl glycerol, and diacyl glycerol. Non-immunogenic, high molecular weight compounds include, but are not limited to, polyethylene glycol, dextran, albumin and magnetite. The nucleic acid ligands described herein can be complexed with a lipophilic compound (e.g., cholesterol) or attached to or encapsulated in a complex comprised of lipophilic components (e.g., a liposome). The complexed nucleic acid ligands can enhance the cellular uptake of the nucleic acid ligands by a cell for delivery of the nucleic acid ligands to an intracellular target. The complexed nucleic acid ligands can also have enhanced pharmacokinetics and stability. U.S. patent application Ser. No. 08/434,465, filed May 4, 1995, entitled "Nucleic Acid Ligand Complexes," which is herein incorporated by reference describes a method for preparing a therapeutic or diagnostic complex comprised of a nucleic acid ligand and a lipophilic compound or a non-immunogenic, high molecular weight compound.

Please replace paragraph [0063] with the following amended paragraph:

[0063] SELEX provides high affinity ligands of a target molecule. This represents a singular achievement that is unprecedented in the field of nucleic acids research. The present invention applies the SELEX procedure to lectin targets. Specifically, the present invention describes the identification of nucleic acid ligands to Wheat Germ Agglutinin, and the selectin ~~selecting~~, specifically, L-selectin, P-selectin and E-selectin. In the Example section below, the experimental parameters used to isolate and identify the nucleic acid ligands to lectins are described.

Please replace paragraph [0071] with the following amended paragraph:

[0071] Therapeutic compositions of the nucleic acid ligands may be administered parenterally by injection, although other effective administration forms, such as intraarticular injection, inhalant mists, orally active formulations, transdermal iontophoresis or suppositories, are also envisioned. One preferred carrier is physiological saline solution, but it is contemplated that other pharmaceutically acceptable carriers may also be used. In one preferred embodiment, it is envisioned that the carrier and the ligand constitute a physiologically-compatible, slow release formulation. The primary solvent in such a carrier may be either aqueous or nonaqueous in nature. In addition, the carrier may contain other pharmacologically-acceptable excipients for modifying or maintaining the pH, osmolarity, viscosity, clarity, color, sterility, stability, rate of dissolution, or odor of the formulation. Similarly, the carrier may contain still other pharmacologically-acceptable excipients for modifying or maintaining the stability, rate of dissolution, release, or absorption of the ligand. Such excipients are those substances usually and customarily employed to formulate dosages for parenteral ~~parental~~ administration in either unit dose or multi-dose form.

Please replace paragraph [0076] with the following amended paragraph:

[0076] 3) rabbit models for hemorrhagic shock (R. K. Winn et al., 1994, Am. J. Physiol. Heart Circ. Physiol. 267, H2391-H2397), ear ischemia reperfusion injury (D. Mihelcic et al., 1994, Bollod 84, 2333-2328) neutrophil rolling on mesenteric venules (A. M. Olofsson et al., Blood 84, 2749-2758), experimental meningitis (C. Granert et al., 1994, J. Clin. Invest. 93, 929-936); lung, peritoneal and subcutaneous bacterial infection (S. R. Sharer et al., 1993, J. Immunol. 151, 4982-4988), myocardial ischemia/reperfusion ~~reperfusion~~ (G. Montrucchio et al., 1989, Am. J. Physiol. 256, H1236-H1246), central nervous system ischemic injury (W. M. Clark et al., 1991, Stroke 22, 877-883);

Please replace paragraph [0105] with the following amended paragraph:

[0105] where L_T is the concentration of initial ligand, K_L is the binding constant of species L to the protein (assuming 1:1 stoichiometry ~~stoechiometry~~) and K_C is the binding constant of species C to the protein (assuming 1:1 stoichiometry ~~stoechiometry~~). Since it is difficult to obtain a direct solution for equation 1, iteration to determine values of [P] to a precision of 1×10^{-15} was used. Using these values of [P], the concentration of protein-ligand complex [PL] as a function

of $[C_T]$ was determined by the following equation:

$$[P_L] = K_L [L_T][P](1 + K_L[P])$$

Please replace paragraph [0113] with the following amended paragraph:

[0113] For rounds 9 through 11 the WGA mass was again reduced ten fold to further increase stringency. The K_d of round 11 RNA was 68 nM. Sequencing of the bulk starting RNA pool and sixth and eleventh round RNA revealed some ~~non-randomness~~ ~~nonrandomness~~ in the variable region at the sixth round and increased ~~non-randomness~~ ~~nonrandomness~~ at round eleven.

Please replace paragraph [0114] with the following amended paragraph:

[0114] To monitor the ~~progress~~ ~~progress~~ of SELEX, ligands were cloned and sequenced from round 6b and round 11. From each of the two rounds, 36 randomly picked clones were sequenced. Sequences were aligned manually and are shown in Table 2.

Please replace paragraph [0127] with the following amended paragraph:

[0127] At 0.5 μ M, RNA ligands 6.8 and 11.20 (SEQ ID NO: 13 and 40) completely inhibit WGA mediated agglutination of sheep erythrocytes (Table 6). Ligand 11.24 (SEQ ID NO: 19) is not as effective, showing only partial inhibition at 2 μ M, the highest concentration tested (Table 6). (GlcNAc)₃ and GlcNAc completely inhibit agglutination at higher concentrations, 8 μ M and 800 μ M, respectively, (Table 6; Monsigny et al., supra). The inhibition of agglutination verifies ~~varifies~~ the proposition that ligands isolated by this procedure will be antagonists of lectin function. Inhibition also suggests that more than one RNA ligand is bound per WGA dimer, since agglutination is a function of multiple carbohydrate binding sites.

Please replace paragraph [0159] with the following amended paragraph:

[0159] For cell binding assays, a constant number of cells were titrated with increasing concentrations of radiolabeled ligand. The test ligands were serially diluted in DPBS(-)/1% BSA to 2-times the desired final concentration approximately 10 minutes before use. Equal volumes (25 μ l) of each ligand dilution and the cell suspension (2×10^7 cells/ml) were added to 0.65 ml eppendorf tubes, gently vortexed and incubated on ice for 30 minutes. At 15 minutes the tubes were revortexed. The ligand/PBMC suspension was layered over 50 μ l of ice cold phthalate oil

(1:1=dinonyl:dibutyl phthalate) and microfuged (14,000 g) for 5 minutes at 4° C. Tubes were frozen in dry ice/ethanol, visible pellets amputated into scintillation vials and counted in Beckman LS6500 scintillation ~~seintillation~~ counter as described in Example 7, paragraph C.

Please replace paragraph [0161] with the following amended paragraph:

[0161] The ability of evolved RNA pools or cloned ligands to inhibit the binding of LS-Rg to sialyl-Lewis^X was tested in competitive ~~competitive~~ ELISA assays (C. Foxall et al., 1992, supra). For these assays, the wells of Corning (25801) 96 well microtiter plates were coated with 100 ng of a sialyl-Lewis^X/BSA conjugate, air dried overnight, washed with 300 µl of PBS(-) and then blocked with 1% BSA in SHMCK for 60 min at room temperature. RNA ligands were incubated with LS-Rg in SHMCK/1% BSA at room temperature for 15 min. After removal of the blocking solution, 50 µl of LS-Rg (10 nM) or a LS-Rg (10 nM)/RNA ligand mix was added to the coated, blocked wells and incubated at room temperature for 60 minutes. The binding solution was removed, wells were washed with 300 µl of PBS(-) and then probed with HRP conjugated anti-human IgG, at room temperature to quantitate LS-Rg binding. After a 30 minute incubation at room temperature in the dark with OPD peroxidase substrate (Sigma P9187), the extent of LS-Rg binding and percent inhibition was determined from the OD₄₅₀.

Please replace the heading **before** paragraph [0162] with the following amended heading:

Example 8

2'-NH₂ NH₂ Ligands to Human L-Selectin

A. SELEX

Please replace paragraph [0163] with the following amended paragraph:

[0163] Binding experiments with 6th round RNA revealed that the affinity of the evolving pool for L-selectin was temperature sensitive. Beginning with round 7, the SELEX was branched; one branch was continued at 4° C. (Table 7a) while the other was conducted at room temperature (Table 7b). Bulk sequencing of 6th, 13th (rm temp) and 14th (4° C.) RNA pools revealed noticeable non-randomness at round six and dramatic non-randomness ~~non-randomness~~ at the later rounds. The 6th round RNA bound monophasically at 4° C. with a dissociation constant of approximately 40 nM, while the 13th and 14th round RNAs bound biphasically with high

affinity Kds of approximately 700 pM. The molar fraction of the two pools that bound with high affinity were 24% and 65%, respectively. The binding of all tested pools required divalent cations. In the absence of divalent cations, the Kds of the 13th and 14th round pools increased to 45 nM and 480 nM, respectively (HSMC, minus $\text{Ca}^{++}/\text{Mg}^{++}$, plus 2 mM EDTA).

Please replace paragraph [0168] with the following amended paragraph:

[0168] The best defined consensus sequences are those of family I, AUGUGUA (SEQ ID NO: 118), and of family III, AACGAAGUA (SEQ ID NO: 120), as shown in Table 8. Family III has two additional, variably spaced sequences, AGUC and ARUAG (SEQ ID NO: 397), that may be conserved. The tetranucleotide AUGW is found in the consensus sequence of families I, III, and VII and in families II, VIII and IX. If this sequence is significant, it suggests that the conserved sequences of ligands of family VIII are circularly permuted. The sequence AGAA is found in the consensus sequence of families IV and VI and in families X and XIII.

Please replace paragraph [0176] with the following amended paragraph:

[0176] Comparative analysis of the family I alignment suggests a hairpin structure in which the consensus sequence, AUGUGUGA (SEQ ID NO: 398), is contained within a variable size loop (FIG. 7a). The stem sequences are not conserved and may be either 5' or 3'-fixed or variable sequence. The one ligand that does not form a stem, F14.25 (SEQ ID NO: 73), has a significantly lower affinity than the other characterized ligands (Table 9).

Please replace paragraph [0177] with the following amended paragraph:

[0177] The proposed structure for family III is also a hairpin with the conserved sequence, AACGAAGUA (SEQ ID NO: 120), contained within a variable length loop (FIG. 7b). The 5'-half of the stem is 5'-fixed sequence which may account in part for the less highly conserved sequence, AGUC.

Please replace paragraph [0181] with the following amended paragraph:

[0181] The SELEX procedure is described in detail in U.S. Pat. No. 5,270,163 and elsewhere. The strategy used for this ssDNA SELEX is essentially identical to that described in Example 7, paragraph B except as noted below. The nucleotide sequence of the synthetic DNA template for

the LS-Rg SELEX was randomized at 40 positions. This variable region was flanked by BH 5' and 3' fixed regions. The random DNA template was termed 40BH (SEQ ID NO: 126) and had the following sequence: 5'-ctacctacgatctgactagc<40N>sgcttactctcatgttagttcc-3' (SEQ ID NO: 126). The primers for the PCR were the following: 5' Primer: 5'-ctacctacgatctgactagc-3' (SEQ ID NO: 127) and 3' Primer: 5'-ajajagggaactacatgagagtaagc-3'; j=biotin (SEQ ID NO: 128). The fixed regions include primer annealing sites for PCR amplification. The initial DNA pool contained 500 pmols of each of two types of single-stranded DNA: 1) synthetic ssDNA and 2) PCR amplified, ssDNA from 1 nmol of synthetic ssDNA template.

Please replace paragraph [0182] with the following amended paragraph:

[0182] For subsequent rounds, eluted DNA was the template for PCR amplification. PCR conditions were 50 mM KCl, 10 mM Tris-Cl, pH 8.3, 7.5 mM MgCl₂, 1 mM of each dATP, dCTP, dGTP, and dTTP and 25 U/ml of the Stoffel fragment of Taq DNA polymerase. After PCR amplification, double stranded DNAs were end-labeled using $\gamma^{32}\text{P}$ -ATP. Complementary strands were separated by electrophoresis through an 8% polyacrylamide 7M urea gel. Strand separation results from the molecular weight difference of the strands due to biotinylation ~~biotinylation~~ of the 3' PCR primer. In the final rounds, DNA strands were separated prior to end labelling in order to achieve high specific activity. Eluted fractions were processed by ethanol precipitation.

Please replace paragraph [0194] with the following amended paragraph:

[0194] A photo-crosslinking version of DNA ligand LD201T4 (SEQ ID NO: 187) was synthesized by replacing nucleotide T15 (FIG. 12) with 5-bromo-deoxyuracil. 4 nmol of ^{32}P -labeled DNA was incubated with 4 nmol L-selectin-Rg in 4 ml 1xSHMCK+0.01% human semi albumin (w/v), then irradiated at ambient temperature with 12,500 pulses from an excimer laser at a distance of 50 cm and at 175 mJ/pulse. Protein and DNA were precipitated with 400 μl 3 M sodium acetate and 8.4 ml ethanol followed by incubation at -70 degrees C. Precipitated material was centrifuged, vacuum dried and resuspended in 100 μl 0.1 M Tris pH 8.0, 10 mM CaCl₂. Forty-five μg ~~[[fig]]~~ chymotrypsin were added and after 20 min at 37 degrees C, the material was loaded onto an 8% polyacrylamide/7M urea ~~polyacrylamide/7M urea~~ 1xTBE gel and electrophoresed until the xylene cyanole had migrated 15 cm. The gel was soaked for 5 min

in 1xTBE and then blotted for 30 min at 200 mA in 1xTBE onto Immobilon-P (Millipore). The membrane was washed for 2 min in water, air dried, and an autoradiograph taken. A labeled band running slower than the free DNA band, representing a chymotryptic peptide crosslinked to LD201T4, was observed and the autoradiograph was used as a template to excise this band from the membrane. The peptide was sequenced by Edman degradation, and the resulting sequence was LEKTLP_SRSYY (SEQ ID NO: 399). The blank residue corresponds to the crosslinked amino acid, F82 of the lectin domain.

Please replace paragraph [0204] with the following amended paragraph:

[0204] Family 1 is defined by ligands from 33 lineages and has a well defined consensus sequence, TACAAGGYGYTAVACGTA (SEQ ID NO: 181). The conservation of the CAAGG (SEQ ID NO: 400) and ACG and their 6 nucleotide spacing is nearly absolute (Table 12). The consensus sequence is flanked by variable but complementary sequences that are 3 to 5 nucleotides in length. The statistical dominance of family 1 suggests that the properties of the bulk population are a reflection of those of family 1 ligands. Note that ssDNA family I and 2'-NH₂ family I share a common sequence, CAAGGCG (SEQ ID NO: 401) and CAAGGYG (SEQ ID NO: 402), respectively.

Please replace paragraph [0205] with the following amended paragraph:

[0205] Family 2 is represented by a single sequence and is related to family 1. The ligand contains the absolutely conserved CAAGG (SEQ ID NO: 400) and highly conserved ACG of family 1 although the spacing between the two elements is strikingly different (23 compared to 6 nucleotides).

Please replace paragraph [0206] with the following amended paragraph:

[0206] Families 4-6 are each defined by a small number of ligands which limits confidence in their consensus sequence, while family 7 is defined by a single sequence which precludes determination of a consensus. Family 5 appears to contain two conserved sequences, AGGGT (SEQ ID NO: 403) and RCACGAYACA (SEQ ID NO: 404), the positions of which are circularly permuted.

Please replace paragraph [0222] with the following amended paragraph:

[0222] Sialyl Lewis^X is the minimal carbohydrate ligand bound by ~~selectin~~ selecting. The ability of ssDNA ligands to inhibit the binding of L-selectin to Sialyl Lewis^X was determined in competition ELISA assays as described in Example 13, paragraph I. LD201T1 (SEQ ID NO: 185), LD174T1 (SEQ ID NO: 194) and LD196T1 (SEQ ID NO: 195) inhibited LS-Rg binding to immobilized SLe^X in a dose dependent manner with IC₅₀s of approximately 3 nM. This is a 10⁵-10⁶-fold improvement over the published IC₅₀ values for SLe^X in similar plate-binding assays. A scrambled sequence based on LD201T1 showed no activity in this assay. These data verify that DNA ligands compete with sialyl-Lewis^X for LS-Rg binding and support the contention that low concentrations of EDTA specifically elute ligands that bind the lectin domain's carbohydrate binding site.

Please replace paragraph [0242] with the following amended paragraph:

[0242] The 30N7 and 40N7 SELEX final pools shared a common major sequence family, even though identical sequences from the two SELEXes are rare (Table 16). Most ligands (72 of the 92 unique sequences) from the 30N7 and 40N7 SELEXes contain one of two related sequence motifs, RYGYGUUUUCRAGY (SEQ ID NO: 405) or RYGYGUUWWUCRAGY (SEQ ID NO: 406). These motifs define family 1. Within the family there are three subfamilies. Subfamily 1a ligands (53/66) contain an additional sequence motif, CUYARRY (SEQ ID NO: 407), one nucleotide 5' to the family 1 consensus motifs. Subfamily 1b (9/66 unique sequences) lacks the CUYARRY (SEQ ID NO: 407) motif. Subfamily 1c (5/66) is also missing the CUYARRY (SEQ ID NO: 407) motif, has an A inserted between the Y and G of consensus YGUU and lacks the consensus GA base pair. The significance of the sequence subfamilies is reflected in the postulated secondary structure of the ligands (Example 25).

Please replace paragraph [0243] with the following amended paragraph:

[0243] A second family, composed of 5 sequences, has a relatively well defined consensus: UACUAN₀₋₁UGURCG (SEQ ID NO: 408) . . . UYCACUAAGN₁₋₂CCC (SEQ ID NO: 409) (Table 16). Family 3 has a short, unreliable consensus motif (Table 16). In addition, there are approximately 12 orphans or apparently unrelated sequences. Three of the orphan sequences were recovered at least twice (Table 16).

Please replace paragraph [0249] with the following amended paragraph:

[0249] The deduced secondary structure of family Ia ligands from comparative analysis of 21 unique sequences is a hairpin motif (FIG. 15) consisting of a 4 to 7 nucleotide terminal loop, a 6 base upper stem and a lower stem of 4 or more base pairs. The consensus terminal loops are either a UUUU tetraloop or a UUWWU (SEQ ID NO: 410) pentaloop. Hexa- and heptaloops are relatively rare. The upper and lower stems are delineated by a 7 nucleotide bulge in the 5'-half of the stem. Four of the six base pairs in the upper stem and all base pairs in the lower stem are supported by Watson-Crick covariation. Of the two invariant base pairs in the upper stem, one is the loop closing GC, while the other is a non-standard GA. The lower stem is most often 4 or 5 base pairs long but can be extended. While the sequence of the upper stem is strongly conserved, that of the lower stem is not, with the possible exception of the YR' base pair adjacent to the internal bulge. This base pair appears to covary with the 3' position of the 7 nucleotide bulge in a manner which minimizes the likelihood of extending the upper stem. Both the sequence (CUYARRY; SEQ ID NO: 407) and length (7 nt) of the bulge are highly conserved.

Please replace paragraph [0281] with the following amended paragraph:

[0281] Family 1 is defined by 23 ligands from 13 independent lineages. The consensus sequence is composed of two variably spaced sequences, CUCAACGAMC (SEQ ID NO: 411) and CGCGAG (SEQ ID NO: 412) (Table 19). In 11 of 13 ligands the CUCAA (SEQ ID NO: 413) of the consensus is from 5' fixed sequence which consequently minimizes variability and in turn reduces confidence in interpreting the importance of CUCAA (SEQ ID NO: 413) or the paired GAG (see Example 27).